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(54) **Bioluminescence measurement system.**

(57) The present invention provides a method of increasing the duration of detectable photon emission of a luciferase-luciferin reaction. The method provides a luciferase-luciferin reaction in which photon emission can be detected for up to and including eight hours. A method of the present invention can also be used to detect the presence of luciferase in biological samples. The present invention also provides a composition used in detecting the presence of luciferase in biological samples.

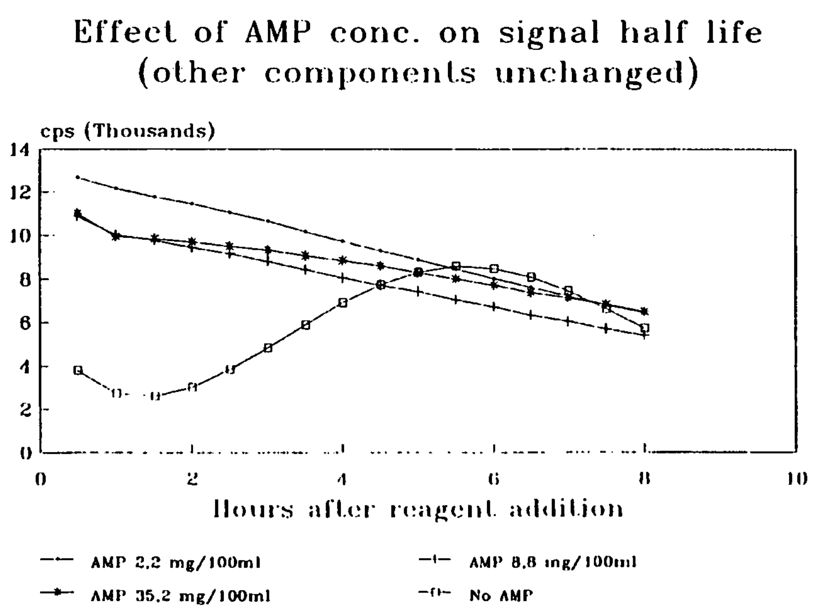


Figure 1

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## Field of the Invention

This invention relates generally to the stabilization of luciferase catalyzed luminescence. The invention relates as well to compositions and methods for improving the lifetimes of the luminescence reaction. This invention also includes assay kits for detection of an expressed luciferase gene used in reporter-gene techniques.

## Background of the Invention

Luciferases are found in a wide variety of organisms including fireflies, photobacteria, jellyfish and many others. Luciferases are enzymes which catalyze the production of light by oxidizing luciferin to oxyluciferin in a process known generally as bioluminescence.

The production of photons by luciferase occurs through a two step reaction which consumes luciferin, adenosine triphosphate (ATP) and $O_2$. In the first step, luciferase catalyzes the formation of luciferyl adenylate from luciferin and ATP. In this first step pyrophosphate is released and a $Mg^{+2}$ cofactor or another divalent cation is required for proper luciferase function. Upon formation, luciferyl adenylate remains within the active site of luciferase. In the next step, luciferase oxidizes luciferyl adenylate to an electronically excited oxyluciferin with the consumption of oxygen. Light production occurs when the electronically excited oxyluciferin decays to the ground state oxyluciferin. The decay from the excited state to the ground state occurs with the concomitant emission of a photon. The color of the light produced differs with the source of the luciferase and appears to be determined by differences in the structure of the various luciferases.

Luciferases have recently become useful in reporter-gene technology. In this technique, a reporter gene, such as a luciferase encoding polynucleotide is used as an indicator for the transcription and translation of a gene in a cellular expression system. The reporter gene is operatively linked to a promoter that is recognized by the cellular expression system. Other commonly used reporter genes include β-galactosidase, and chloramphenicol acetyltransferase (CAT). In a typical reporter gene assay, a DNA vector containing the reporter gene is transfected into a cell capable of expressing the reporter gene. After a sufficient amount of time to allow for the expression of the reporter gene has passed, the cellular membrane is disrupted to release the expressed gene product. The reagents necessary for the catalytic reaction of the reporter gene are then added to the reaction solution and the enzymatic activity of the reporter gene is determined. Alternatively, the cell can be disrupted in the presence of all reagents necessary for the determination of the enzymatic activity of the reporter gene. If a β-galactosidase encoding polynucleotide is used as the reporter gene, the hydrolysis of a galactoside is determined. If a chloramphenicol acetyltransferase encoding polynucleotide is used as the reporter gene, the production of an acetylated chloramphenicol is determined. When luciferase is used as the reporter gene the photons produced from the luciferase-luciferin reaction is measured.

A major problem in determining expression of a luciferase gene as a reporter gene is the short duration of photon production. Typically, luciferase catalyzed photon production ceases within a few seconds. Means for extending the period of photon production have been eagerly sought. Currently a commercially available kit from the Promega Corporation (Madison, Wi) can extend the half-life of luciferase catalyzed photon production to roughly five minutes. Nevertheless, for the measurement of large numbers of samples, luciferase catalyzed photon production with a half-life of only five minutes is not a viable alternative. As used herein, half-life is the time it takes for photon production to decrease by one half.

The present invention provides methods and compositions for increasing the duration of detectable photon emission of a luciferase-luciferin reaction.

## Brief Summary of the Invention

In one aspect, the present invention provides a method for increasing the duration of detectable photon emission of a luciferase-luciferin reaction. In one embodiment, a reaction mixture containing luciferase, luciferin, ATP, and cofactors required for luciferase catalytic activity is mixed with a composition containing adenosine monophosphate, a radical scavenger and a chelating agent to form an admixture. The photons produced by the luciferase-luciferin reaction is then detected by measuring the luminescence of the admixture. The luciferase catalyzed photon production can be detected for more than five minutes.

In a preferred embodiment, 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate (ATP), cofactors necessary for luciferase catalytic activity, about 2.2 mg adenosine monophosphate (AMP), about 385 mg dithiothreitol (DTT), and about 20 mg ethylenediaminetetraacetic and (EDTA).

In another embodiment, the amount of one of the components of the admixture can be varied while the amounts of all other components remain unchanged. For example, in 100 ml of the admixture, the amount of luciferin can be varied between about 0.2 to about 30 mg while maintaining the amount of ATP at about 110mg, AMP at about 2.2 mg, DTT at about 385 mg and EDTA at about 20 mg. Similarly, components can be varied individually as follows: for 100 ml of the admixture, the amount of luciferin can be varied from about 0.2 to about 30 mg; the amount of ATP can be varied from about 10 to about 300 mg; the amount of AMP can be varied from about 0.2 to about 30 mg; the amount DTT can be varied from about 200 to about 2000 mg; and the amount EDTA can be varied from between about 10 to about 50 mg.

In another embodiment, the present invention contemplates a method for detecting the presence of luciferase in a biological sample. The biological sample suspected of containing luciferase is mixed with a reaction mixture which contains luciferin, adenosine triphosphate, cofactors required for luciferase catalytic activity, adenosine monophosphate, dithiothreitol, ethylenediaminetetraacetic acid, phenylacetic acid, oxalic acid, and a detergent to form an admixture. The photons produced by the luciferase-luciferin reaction are then detected by measuring the luminescence of the admixture.

In a preferred embodiment, the present invention contemplates an admixture for detecting the presence of a luciferase in a biological sample. One hundred ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, and about 0.85 mg oxalic acid.

In another embodiment, the amount of one of the components of the admixture can be varied while the amounts of all other components remain unchanged. For example, in 100 ml of the admixture, the amount of luciferin can be varied between about 0.2 to about 30 mg while maintaining the amount of ATP at about 110mg, AMP at about 2.2 mg, DTT at about 385 mg, EDTA at about 20 mg, phenylacetic acid at about 4.5 mg, and oxalic acid at about 0.85 mg. Similarly, components can be varied individually as follows: for 100 ml of the admixture, the amount of luciferin can be varied from about 0.2 to about 30 mg; the amount of ATP can be varied from about 10 to about 300 mg; the amount of AMP can be varied from about 0.2 to about 30 mg; the amount DTT can be varied from about 200 to about 2000 mg; the amount of EDTA can be varied from about 10 to about 50 mg; the amount of phenylacetic acid can be varied from about 1 to about 10 mg; and the amount of oxalic acid can be varied from about 0.2 to about 5 mg.

In another aspect, the present invention contemplates a composition used in detecting the presence of luciferase in biological samples by detecting a emitted photon from a luciferase-luciferin reaction. One hundred ml of this composition contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, about 0.85 mg oxalic acid, and about 4 g triton.

## Brief Description of the Drawings

Figure 1 shows the effect of varying the AMP concentration on luciferase-luciferin light production.

Figure 2 shows the effect of varying DTT concentration on luciferase-luciferin light production.

Figure 3 shows the effect of varying both DTT and AMP concentration on luciferase-luciferin light production.

Figure 4 shows the effect of various reagents to the luciferase-luciferin reaction.

Figure 5 shows the light production of a reporter-gene assay using a cloned luciferase.

Figure 6 shows that Compound A is either an unspecific inhibitor of the HIV-rev regulatory protein or is toxic to the cells.

Figure 7 shows that Compound B is a specific inhibitor of the HIV-rev regulatory protein.

## Detailed Description of the Invention

Luciferases catalyze the oxidation of luciferin with the concomitant emission of photons. The present invention provides compositions and methods for increasing the duration of detectable photon emission of a luciferase-luciferin reaction. The present invention provides a luciferase-luciferin reaction in which light production can be detected for more than five minutes. In a preferred embodiment, the photon emission of a luciferase-luciferin reaction can be detected for up to and including eight hours. In another embodiment, detectable photon emission from a luciferase-luciferin reaction is linear for up to eight hours (see Figures 1-5).

In one embodiment, a reaction mixture containing luciferase, luciferin, ATP, and cofactors required for luciferase catalytic activity is mixed with a composition containing adenosine monophosphate, a radical scavenger and a chelating agent to form an admixture. The photons produced by the luciferase-luciferin reaction are then detected by measuring the luminescence of the admixture. Luciferase catalyzed photon emission as disclosed by the methods and compositions of the present invention can be detected for more than five minutes. In a preferred embodiment, photon emission can be detected for more than thirty minutes and for up to eight hours. In a preferred embodiment, photon emission decays linearly for up to eight hours as shown in Figures 1-3.

In a preferred embodiment, the present invention provides a luciferase-luciferin reaction admixture. One hundred ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, and about 20 mg ethylenediaminetetraacetic acid.

In another embodiment, the amount of one of the components of the admixture can be varied while the amounts of all other components remain unchanged. For example, in 100 ml of the admixture, the amount of luciferin can be varied between about 0.2 to about 30 mg while maintaining the amount of ATP at about 110mg, AMP at about 2.2 mg, DTT at about 385 mg and EDTA at about 20 mg. Similarly, components can be varied individually as follows: for 100 ml of the admixture, the amount of luciferin can he varied from about 0.2 to about 30 mg; the amount of ATP can be varied from about 10 to about 300 mg; the amount of AMP can be varied from about 0.2 to about 30 mg; the amount DTT can be varied from about 200 to about 2000 mg; and the amount of EDTA can be varied from between about 10 to about 50 mg. As an example, the present invention contemplates an admixture of the following composition: 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, between about 0.2 to about 35 mg adenosine monophosphate, about 385 mg dithiothreitol, and about 20 mg ethylenediaminetetraacetic acid. Other admixtures in which only one component is varied in accordance with the limitations disclosed above are contemplated.

In another aspect, the present invention contemplates a method for detecting the presence of luciferase in a biological sample. The biological sample suspected of containing luciferase is mixed with a reaction mixture which contains luciferin, adenosine triphosphate, cofactors required for luciferase catalytic activity, adenosine monophosphate, dithiothreitol, ethylenediaminetetraacetic acid, phenylacetic acid, oxalic acid, and a detergent to form an admixture. The photons produced by the luciferase-luciferin reaction is then detected by measuring the luminescence of the admixture. A preferred biological sample is a cell that produces luciferase. An exemplary detergent is triton N101. The present invention provides methods and compositions in which the presence of luciferase in the biological sample can be detected for more than five minutes by detection of the emitted photon. In a preferred embodiment, photon emission can be detected for more than thirty minutes and for up to eight hours. In a preferred embodiment, photon emission decays linearly for up to eight hours as shown in Figures 1-3.

In a preferred embodiment, the present invention contemplates an admixture for detecting the presence of luciferase in a biological sample. One hundred ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, about 0.85 mg oxalic acid, and a detergent.

In another embodiment, the amount of one of the components of the admixture can be varied while the amounts of all other components remain unchanged. For example, in 100 ml of the admixture, the amount of luciferin can be varied between about 0.2 to about 30 mg while maintaining the amount of ATP at about 110mg, AMP at about 2.2 mg, DTT at about 385 mg, EDTA at about 20 mg, phenylacetic acid at about 4.5 mg, and oxalic acid at about 0.85 mg. Similarly, components can be varied individually as follows: for 100 ml of the admixture, the amount of luciferin can be varied from about 0.2 to about 30 mg; the amount of ATP can be varied from about 10 to about 300 mg; the amount of AMP can be varied from about 0.2 to about 30 mg; the amount DTT and be varied from about 200 to about 2000 mg; the amount of EDTA can be varied from about 10 to about 50 mg; the amount of phenylacetic acid can be varied from about 1 to about 10 mg; and the amount of oxalic acid can be varied from about 0.2 to about 5 mg.

In another aspect, the present invention contemplates a composition used in detecting the presence of luciferase in biological samples by detecting emitted photons from a luciferase-luciferin reaction. One hundred ml of this composition contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, and about 0.85 mg oxalic acid.

In a more preferred embodiment, the present invention contemplates a reaction mixture for detecting the presence of a luciferase in a biological sample, 100 ml of which contains about 2.8 mg luciferin, about

110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, about 0.85 mg oxalic acid, and a detergent. As an example, the present invention contemplates an admixture of the following composition: 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, between about 200 to 2000 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, and about 0.85 mg oxalic acid. Other admixtures in which only one component is varied as discussed above are contemplated.

As used herein, the term "luciferase" is an enzyme which catalyzes the oxidation of luciferin with the concomitant emission of a photon. Luciferases can be isolated from biological specimens which produce luciferase or from a cell which has been transformed or transfected with a polynucleotide encoding for a luciferase. It is within the skill of one of ordinary skill in the art to isolate a luciferase from a biological specimen that produces luciferase. Similarly, means of transforming or transfecting a cell with a polynucleotide that encodes for a luciferase are well known in the art.

One aspect of the invention provides a method for increasing the duration of detectable photon emission of a luciferase-luciferin reaction for more than thirty minutes by the addition of certain reagents. A large number of substances which influence the luciferin-luciferase reaction including dithiothreitol, cytidine nucleotides, AMP, pyrophosphate, coenzyme A, EDTA, protease inhibitors, and luciferase inhibitors including luciferin anologs have been reported.

Decomposition and activation of luciferase decreases the lifetime of the luciferase-luciferin reaction by inactivation of luciferase. In reporter gene techniques where cell lysates are used, the inactivation of luciferase by endogenous proteases present in the cell lysate is a particular problem. The addition of protease inhibitors such as phenylacetic acid (PAA), oxalic acid, monensine, acetyl phenylalanine, leupeptine, ammonium chloride, aprotinin and others prevent the degradation of luciferase by endogenous proteases present in the biological sample. By slowing down or preventing the inactivation of luciferase, luciferase catalyzed light production is lengthened. The above list is illustrative only and many other protease inhibitors are well known in the art. The use of other protease inhibitors is contemplated by the present invention.

The addition of luciferin analogs and other inhibitors of luciferase increase the lifetime of light production by inhibiting luciferase. Care must be taken to ensure that the inhibitor binds to luciferase reversibly. Analogs that bind irreversibly permanently inhibit luciferase. Exemplary reversible inhibitors of luciferase include phenylbenzothiazol, 2-aminoethanol, benzothiazole, 2-hydroxyphenylbenzothiazole and pyrophosphate.

Adenosine monophosphate (AMP) is a catalytic product of the luciferase-luciferin reaction. The addition of AMP promotes the initial phase of the luciferase luciferin reaction. As shown in Figure 1, in the absence of AMP, initial photon emission is low and maximum light production occurs nearly six hours after the initiation of the luciferase-luciferin reaction. In contrast, when the concentration of AMP is between about 2.2 and about 35.2 mg/100 ml, maximum light production occurs immediately and decreases in a linear manner for more than eight hours. Figure 1 shows the effect of varying the AMP concentration on luciferase-luciferin light production. 100 ml of the admixture contained 110 mg adenosine triphosphate, 385 mg dithiothreitol, 2.8 mg luciferin, 20 mg ethylenediaminetetraacetic acid, 4 ml of a 10% triton N101 solution in $H_2O$, 4.5 mg phenylacetic acid, and 0.85 mg oxalic acid in 50 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH, 7.8. The amount of AMP added to 100 ml of the assay solution was varied from about 0 to about 35.2 mg. With the addition of about 8.8 mg and about 35.2 mg of AMP, detectable photon emission was linear between one and eight hours. The addition of 2.2 mg of AMP resulted in photon emission which was linear for up to eight hours.

Thiol compounds such as dithiothreitol (DTT), dithioerythritol, glutathione and other well known reducing agents are radical scavengers and increase the duration of detectable photon emission of a luciferase-luciferin reaction. While the mechanism of thiol compound stabilization of the luciferase-luciferin reaction is not well understood, these compounds probably function by limiting the availability of oxygen necessary in the second step of the luciferase-luciferin reaction. At higher concentrations, DTT increases emission of photons through an unknown mechanism. Figure 2 shows the effect of varying DTT concentration on luciferase-luciferin light production. in Figure 2, 100 ml of the assay solution contained 2.2 mg adenosine monophosphate, 110 mg adenosine triphosphate, 2.8 mg luciferin, 20 mg ethylenediaminetetraacetic acid, 4 ml of a 10% triton N101 solution in $H_2O$, 4.5 mg phenylacetic acid, and 0.85 mg oxalic acid in 50 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.8. The amount of DTT in the admixture varied from 193 to 770 mg. The addition of 385 and 770 mg of DTT resulted in detectable photon emission which was linear for up than eight hours. When 193 mg of DTT is added, light production was reduced and was linear between two and eight hours.

Figure 3 shows the effect of varying both DTT and AMP concentration on the duration of detectable photon emission from a luciferase-luciferin reaction. 100 ml of the admixture contained 110 mg adenosine triphosphate, 2.8 mg luciferin, 20 mg ethylenediaminetetraacetic acid, 4 ml of a 10% triton N101 solution in $H_2O$, 4.5 mg phenylacetic acid, and 0.85 mg oxalic acid, in 50 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH, 7.8. The addition of 385 mg DTT and 2.2 mg AMP resulted in detectable photon emission which was linear between 1.75 and 8 hours. The addition of 770 mg DTT and 35 mg AMP resulted in detectable photon emission which was linear for up to eight hours.

Chelating agents which bind metal ions can increase the duration of photon emission from a luciferase-luciferin reaction by binding $Mg^{+2}$ and other divalent cations including $Ca^{+2}$, $Fe^{+2}$, $Mn^{+2}$, $Co^{+2}$, and $Zn^{+2}$. In reporter gene techniques, the concentrations of divalent cations cannot be rigorously controlled because whole cell lysates are used. Lysis of cells expressing a luciferase encoding gene release all of the endogenous divalent cations including $Mg^{+2}$ and $Ca^{+2}$ into the luciferase-luciferin reaction admixture. The addition of chelating agents effectively removes the released $Mg^{2+}$ and $Ca^{+2}$. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), and ethylene glycol- bis ($\beta$-aminoethyl ether) N,N,N',N',-tetracetic acid (EGTA). The use of other chelating agents are contemplated by the present invention.

The use of detergents to lyse cells suspected of expressing luciferase is well known in the art. Exemplary anionic detergents include the series of triton detergents including triton N-101. The substitution of other detergents, including both ionic and anionic detergents is within the skill of an ordinary artisan.

The use of reagents to maintain the pH of the luciferase-luciferin reaction solution is well known in the art. An exemplary buffering reagent is N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES). Many other buffering reagents are well known and are commercially available.

The present invention provides a combination of reagents which increase the duration of detectable photon emission from a luciferase-luciferin reaction. Figure 4 shows the effect on the lifetime of the luciferase-luciferin reaction in an admixture consisting of luciferase, luciferin, ATP, cofactors required for luciferase catalytic activity and EDTA (20 mg/100 ml) in 50 mM HEPES and AMP, DTT or coenzyme A. In all of the combinations shown in Figure 4, the detectable photon emission from a luciferase-luciferin reaction lasts for at least for eight hours with the exception of a solution containing EDTA and 250 $\mu$M coenzyme A in which photons can be detected for 7 hours.

The linearity in the decrease of photon emission during the life of a luciferase-luciferin reaction is an important aspect of the present invention. Because photon emission is linear during the life of the luciferase-luciferin reaction, initial luminescence can be calculated easily from the luminescence detected at ay time during the life of the luciferase-luciferin reaction. From the calculated initial luminescence, the concentration of the luciferase in the measured sample can be determined. For example, in the reporter gene technique, samples suspected of expressing luciferase can be measured over a period of many hours and the initial concentration of luciferase in the samples can be determined.

As discussed below in Examples 1 and 2 the present invention allows for the measurement of large numbers of samples. Recently, instrumentation for measuring photon emission from 96 well microtiter plates became available ("TopCount Microplate Scintillation and Luminescence Counter" from the Packard Instrument Company, Inc. of Downers Grove, Illinois). The light output from each 96 well plate takes roughly ten minutes to measure. Thus with a linear emission of photons of eight hours, forty eight 96-well plates can be easily measured. This results in the measurement of photon emission from 4608 individual samples.

Example 1: Reporter-gene Assay Using a Luciferase cDNA

The reporter gene technique using a clone luciferase was used to demonstrate the increased duration of the luciferase-luciferin reaction. The production of light from transiently transformed human T-cells (Jurkat) was measured for eight hours. Jurkat cells in the logarithmic phase of growth were transformed by the DEAE-dextran technique and incubated in a humidified incubator for 48 hours under assay conditions. The plasmid vector contained a luciferase gene under the control of the EF promoter. After 48 hours, 100 $\mu$l of the cell suspension was mixed with 100 $\mu$l of PBS and 100 $\mu$l a 100 ml solution containing 2.2 mg adenosine monophosphate, 110 mg adenosine triphosphate, 385 mg dithiothreitol, 2.8 mg luciferin, 20 mg ethylenediaminetetraacetic acid, 4 ml of a 10% triton N101 solution in $H_2O$, 4.5 mg phenylacetic acid, 0.85 mg oxalic acid and 50 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.8. The production of light was then measured for up to eight hours. The "high activity" curve (solid line) represents light production from the transformation of Jurkat cells with 2 $\mu$g of the luciferase containing vector at a concentration of 1 X $10^6$ cells/ml. The "low activity" curve represents light production from the same experiment but with a cellular concentration of 50,000 cells/ml. The half life of the "high activity" sample was found to be roughly 3.5 hours and the half life of the "low activity" curve was found to be roughly 5 hours.

Example 2: High-capacity Luciferase Reporter Gene Assay for HIV-Rev-RRE-Interaction

A transient transfection assay using the DEAE-dextran technique for T-cell lines was established to screen for inhibitors of the HIV-rev regulatory protein (RRE). Two expression vectors, one for the HIV-1 gene (pEF-cRev) and the other for a luciferase reporter gene (pCMV-Luc/RRE) are cotransfected in the Jurkat cell line (lymphoblastoid cells). RRE is expressed together with Luciferase if a certain level of functional rev protein has been accumulated within a cell. The assay mimics the natural action of rev. Rev transports unspliced or singly spliced RRE-containing mRNA out of the nucleus to the translation machinery in the cytoplasm. If a certain threshold level of rev protein accumulates in the nucleus, rev binds to RRE containing mRNA, multimerizes and mediates transport of this mRNA into a cellular pathway bypassing the cellular splicing machinery. This is accomplished by binding of the Rev/RRE complex to a cellular factor, translation initiation factor eIF-5a, of the preribosomes formed within the nucleoli. Inhibitors of any of these steps in the mode of action of rev is detectable in this reporter gene assay.

The assay conditions have been optimized to obtain a stimulation of luciferase expression by rev to approx. 20-100 fold over background within 20 hours of transfection. The basal luciferase expression as determined in control transfections with an identical expression vector carrying the rev gene in antisense orientation to the promoter (pEF-AScRev) is usually below 5% of the rev-induced expression. The toxicity of candidate compounds is determined by a parallel transfection assay. In this assay, a rev-independent luciferase construct (pCMV-LUC) is cotransfected with the rev-expression vector )pEF-cRev) to keep conditions similar to the rev-assay. This toxicity assay also identifies compounds which are inhibitors of the luciferase enzyme or to transcription factors binding to the CMV-promoter. Therefore, the toxicity assay is also capable of acting as a specificity control.

The assay has been adapted to microplate technology and can be automated. While detectors to measure multiple samples have been known for some time, modern single photon measurement techniques with an extraordinarily high sensitivity for detection of bioluminescence signals from microplates came to the market recently. The stabilization of the bioluminescence signal from a half-life of a few minutes to more than 4 hours allows for the automation of the assay by using the newly available microplate single photon counters. A cDNA encoding for a luciferase is therefore an ideal reporter gene. The use of a luciferase is applicable to many other mechanism-based cellular test systems for pharmaceutical, toxicological and other screening systems.

Jurkat T-cell suspension cultures, stock 1722 are split daily with a dilution of at least 1:2. Final cell density should not exceed $6x10^5$ cells/ml to keep the cells in the logarithmic growth phase. Cells can be seeded at a density $0.5x10^5$/ml or lower for maintaining stock cultures. Culturing in roller bottles (approx. 10 rpm) is the preferred culture method.

The following buffers and reagents are used:

- Dulbecco's PBS (phosphate buffered saline)
- DEAE-Dextran (MW ca. 500.000), PHARMACIA # 17-0350-00 solved at 10 mg/ml in PBS and filtered through 0.2 µm Nalgene filters.
- Assay medium: RPMI 1640 without phenol red supplemented with:
  - + 10% FCS inactivated
  - + 1% v/v HEPES buffer (1 M)
  - + 1% Penicillin/Streptomycin, GIBCO 043-05070
  - + 0.1% Gentamicin, GIBCO 043-05750
  - + 1% Chloroquine (10mM = 5.52 mg/ml in PBS), Sigma #C6628
- Wash medium: RPMI 1640 without phenol red supplemented with:
  - + 1% v/v HEPES buffer (1 M)
  - + 1% Penicillin/Streptomycin, GIBCO 043-05070
  - + 0.1% Gentamicin, GIBCO 043-05750
- DNA preparations, purified by two cycles of CsCl gradient centrifugation and stored frozen in aliquots at -20° C (dissolved at 1 mg/ml in $H_2O$)
  - pCMV-Luc/RRE DNA, # 18xx
  - pEF-cREV DNA, # 21xx
  - pEF-AScREV DNA, # 22xx
  - pCMV-Luc DNA, # 3xx
- luciferase-substrate solution:
  - 2.2 mg AMP (Sigma A1877)

110 mg ATP (Sigma A5394)
385 mg Dithiothreitol
280 µl Luciferin (10 mg/ml in $H_2O$), Sigma L5256 dissolved in 100 ml buffer consisting of:
5 ml HEPES (1M)
20 mg EDTA (Titriplex III)
4 ml TRITON N101 (10% in $H_2O$)
4.5 mg Phenylacetic acid, Sigma No. P4514 0.85 mg Oxalic acid, Sigma No. 07626
mg adjusted to 7.8 (2N NaOH, ca 1.1 ml)

- Microplates:
  Packard CulturPlates 6005180 with adhesive seals
- Cellfilter Falcon #2340

The assay protocol is as follows:

Preparation of Dilutions:

Pure substances CHC (500µM in DMSO saturated $H_2O$)) 1:50 final dilution:

- Medium = RPMI 1640 plus additives (see above)
- Predilution: 8 µl sample plus 192 µl assay medium (Microplate)
- Add 50 µl predilution each in parallel positions to 3 white Packard CulturPlates (B1 through H12)
- Blank: 50 µl medium (A1 through A12)
- Plates are kept in a $CO_2$-cabinet until addition of coils

Broth of Actinomycetes at 1:200, Fungi at 1:100 and Bacteria at 1:100 final dilutions:

- Predilution: 8 µl sample plus 392 µl assay medium = 1:50 (Titertek racks), for bacteria and fungi
  8 µl sample plus 792 µl assay medium = 1:100 (Titertek racks), for actinomycetes
- Add 150 µl of predilution each in 3 parallels to white Packard CulturPlates (B1 to H12)
- Blank: 50 µl assay medium (A1 to A12)
- Plates are kept in a $CO_2$-cabinet until addition of cells

Designation of control wells in the primary screen:

| | | |
|---|---|---|
| A1, A2 | plate control | medium only, no cells |
| A3 to A10 | 'high' control | pCMV-Luc/RRE + pEF-cREV |
| A11, A12 | plate control | medium only, no cells |

Secondary screening (validation) of the positive/toxic hits (1:50 to 1:6400 final dilution for substances):

- Predilution: 20 µl sample plus 480 µl assay medium (Titertek racks)
- Add 100 µl predilution in duplicates to the wells of rows A (3 to 12) to two Packard CulturPlates in parallel
- Add 50 µl of assay medium to all the remaining wells
- Dilute columns 3 to 12 by serially transferring 50 µl and discarding the rest after row H.

Preparation of Cells and Transfection Procedure:

- Cells as described above, are centrifuged (200 g, 10 min) and washed twice with prewarmed wash medium.
- Cells are suspended in prewarmed PBS and counted in a hemacytometer; the cell density is adjusted to $4 \times 10^6$ cells/ml.
- DNA-solutions are prepared in prewarmed PBS (2.5 ml/plate, 25 µl/well to be tested):
  - Add 25 µl DEAE/Dextran pr ml PBS.
  - Add DNA:
    - For REV/RRE: 0.60 µl/ml pCMV-Luc/RRE # 18xx and 1 µl/ml pEF-cREV # 21xx.

- For negative controls: 0.60 µl/ml pCMV-Luc/RRE # 18xx and 1 µl/ml AScREV # 22xx.
- For toxicity/unspecific inhibition: 0.60 µl/ml pCMV-Luc # 3xx and 1µl/ml pEF-cREV # 21 xx.
- For toxicity/handling control: 0.60 µl/ml pCMV-Luc # 3xx and 1 µl/ml pEF-AScREV # 22xx.
- Add an equal volume of cell suspension ($4x10^6$ cells/ml) to each of the DNA-solutions.

- The DNA/cell suspension is incubated for 10 min. at 37°, shaken gently by hand and incubated for another 10 min.
- An equal volume of prewarmed assay medium is added, cells are shaken gently by hand and incubated for another 10 min.
- The pelleted cells are resuspended in prewarmed RPMI 1640 plus additives (assay medium) and adjusted to a final concentration of $1x10^6$ cells/ml.
- Cells are sucked once through a syringe and filtered through a cell filter immediately before being dispensed to the plates.
- 50 µl of cell suspension is added to each of the respective wells:

  Secondary Jurkat-Rev Assay:
  - 'High' control and substance dilutions: pCMV-luc/RRE + pEF-cRev in wells A1-H1
  - Blanks: 50 µl medium in wells A2-D2
  - 'Low' control: pCMV-luc/RRE + pEF-AScRev in wells E2-H2

  Secondary Jurkat-Toxicity Assay:
  - 'High' control and substance dilutions: pCMV-luc + pEF-cRev in wells A1-H1
  - Blanks: 50 µl medium in wells A2-D2
  - 'Handling' control: pCMV-luc + pEF-AScRev in wells E2-H2
- Plates are shaken using a microplate-shaker all stored in the incubator at 37° C in a 5% $CO_2$ atmosphere for 16 - 24 hours.

Measurement of Luciferase Activity

- After incubation, 100 µl of luciferase substrate solution, to be prepared freshly every day, is added to each well.
- Plates are shaken using a microplate shaker for a few seconds and sealed with an adhesive seal (do NOT use heat-seals).
- Plates are counted in a Packard "TopCount Microplate Scintillation and Luminescence Counter" or an equivalent instrument after a count delay of 2 min. for 0.15 min. per well. The temperature of the counting chamber as well as the plate stack should not exceed 22° C, otherwise the half life of the light emission can drop below 4 hours.
- The REV-Luc/RRE ('high'-control) - and the Luc (toxicity-control) - background corrected valued should be at last 2000 cps, the AsREV-Luc/RRE ("low"-control) should not exceed 100 cps.

Results

Figures 6 and 7 show the results of a large scale compound screening test for compounds which inhibit HIV-rev regulatory protein (RRE). Jurkat cells are co-transfected with two plasmids, pEF-cRev and either pCMV-Luc/RRE or pCMV-Luc. The closed square represents cells which have been transfected with pEF-cREV and pCMV-Luc/RRE. The open square represents cells which have been transfected with pEF-cREV and pCMV-Luc. If a candidate compound inhibits RRE, cells transfected with pEF-cREV ad pCMV-Luc/RRE should show a decrease in light production from the luciferase-luciferin reaction compared to cells transfected with pEF-cREV and pCMV-Luc. If a candidate compound does not inhibit RRE, then light production from the luciferase-luciferin reaction should remain unchanged whether cells are transfected with pCMV-Luc/RRE or pCMV-Luc.

Figure 6 shows the effect of Compound A on RRE. The inhibition curves obtained from cells co-transfected with pEF-cRev and either pCMV-Luc/RRE (closed square) or with pCMV-Luc (open square) is not dramatically different. Compound 6 therefore does not appear to be an inhibitor of RRE. Alternatively, the toxicity of Compound A may prematurly kill the transfected cells before meaningful data can be obtained.

Figure 7 shows the effect of Compound B on RRE. The inhibition curves obtained from cells co-transfected with pEF-cRev and either pCMV-Luc/RRE (closed square) or with pCMV-Luc (open square) is dramatically different. There is a significant difference in inhibition of RRE between cells transfected with pCMV-Luc/RRE and cells transfected with pCMV-Luc. In cells transfected with pCMV-Luc/RRE, about 0.1 µM Compound B results in 50% inhibition. In cells transfected with pCMV-Luc, Compound B must be present in concentrations greater than 10 µM to obtain 50% inhibition (data not shown).

**Claims**

1. A method for increasing the duration of detectable photon emission of a luciferase-luciferin reaction mixture containing luciferase, luciferin adenosine triphosphate, and cofactors necessary for luciferase catalytic activity, the method comprising mixing the reaction mixture with a composition comprising adenosine monophosphate, a free radical scavenger and a chelating agent to form a admixture.

2. The method of Claim 1, wherein the free radical scavenger is dithiothreitol.

3. The method of Claim 1, wherein the chelating agent is ethylenediaminetetraacetic acid.

4. The method of Claim 1, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, and about 20 mg ethylenediaminetetraacetic acid.

5. The method of Claim 1, wherein 100 ml of the admixture contains between about 0.2 to about 30 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, and about 20 mg ethylenediaminetetraacetic acid.

6. The method of Claim 1, wherein 100 ml of the admixture contains about 2.8 mg luciferin, between 10 to about 300 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, and about 20 mg ethylenediaminetetraacetic acid.

7. The method of Claim 1, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, between about 0.2 to about 35 mg adenosine monophosphate, about 385 mg dithiothreitol, and about 20 mg ethylenendiaminetetraacetic acid.

8. The method of Claim 1, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2. mg adenosine monophosphate, between about 200 to 2000 mg dithiothreitol, and about 20 mg ethylenediaminetetraacetic acid.

9. The method of Claim 1, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, and between about 10 to 50 mg ethylenediaminetetraacetic acid.

10. A method for detecting the presence of luciferase in a biological sample comprising:
    (a) mixing a sample suspected of containing luciferase with a reaction mixture containing luciferin, adenosine triphosphate, cofactors necessary for luciferase catalytic activity, adenosine monophosphate, a protease inhibitor, a free radio scavenger, a chelating agent, and a detergent to form an admixture and
    (b) measuring the luminescence.

11. The method of Claim 10, wherein the free radical scavenger is dithiothreitol.

12. The method of Claim 10, wherein the chelating agent is ethylenediaminetetraacetic acid.

13. The method of Claim 10, wherein the protease inhibitor is phenylacetic acid or oxalic acid.

14. The method of claim 10, wherein the detergent is triton N-101.

15. The method of Claim 10, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, and about 0.85 mg oxalic acid.

16. The method of Claim 10, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, between about 1 to about 10 mg phenylacetic acid, and about 0.85 mg oxalic acid.

17. The method of Claim 10, wherein 100 ml of the admixture contains about 2.8 mg luciferin, about 110 mg adenosine triphsophate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 phenylacetic acid, and between about 0.2 to about 5 mg oxalic acid.

18. The method of claim 10, wherein the biological sample comprises a cell that produces luciferase.

19. A composition used in detecting the presence of luciferase in biological samples wherein 100 ml of the composition contains about 2.8 mg luciferin, about 110 mg adenosine triphosphate, about 2.2 mg adenosine monophosphate, about 385 mg dithiothreitol, about 20 mg ethylenediaminetetraacetic acid, about 4.5 mg phenylacetic acid, about 0.85 mg oxalic acid, and about 4 g triton.

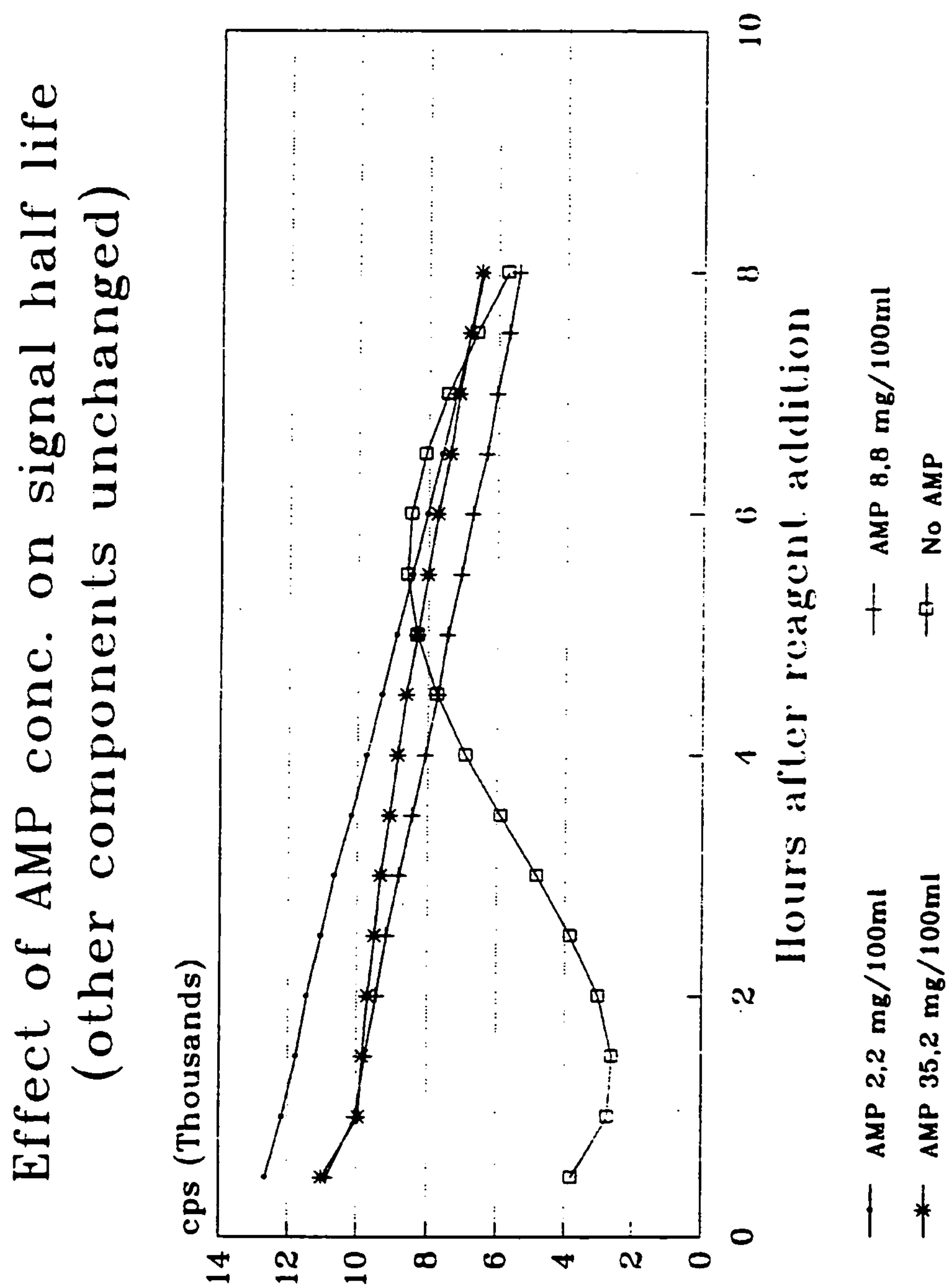

Effect of AMP conc. on signal half life
(other components unchanged)
cps (Thousands)
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Hours after reagent addition
AMP 2,2 mg/100ml
AMP 35,2 mg/100ml
AMP 8,8 mg/100ml
No AMP

Figure 1

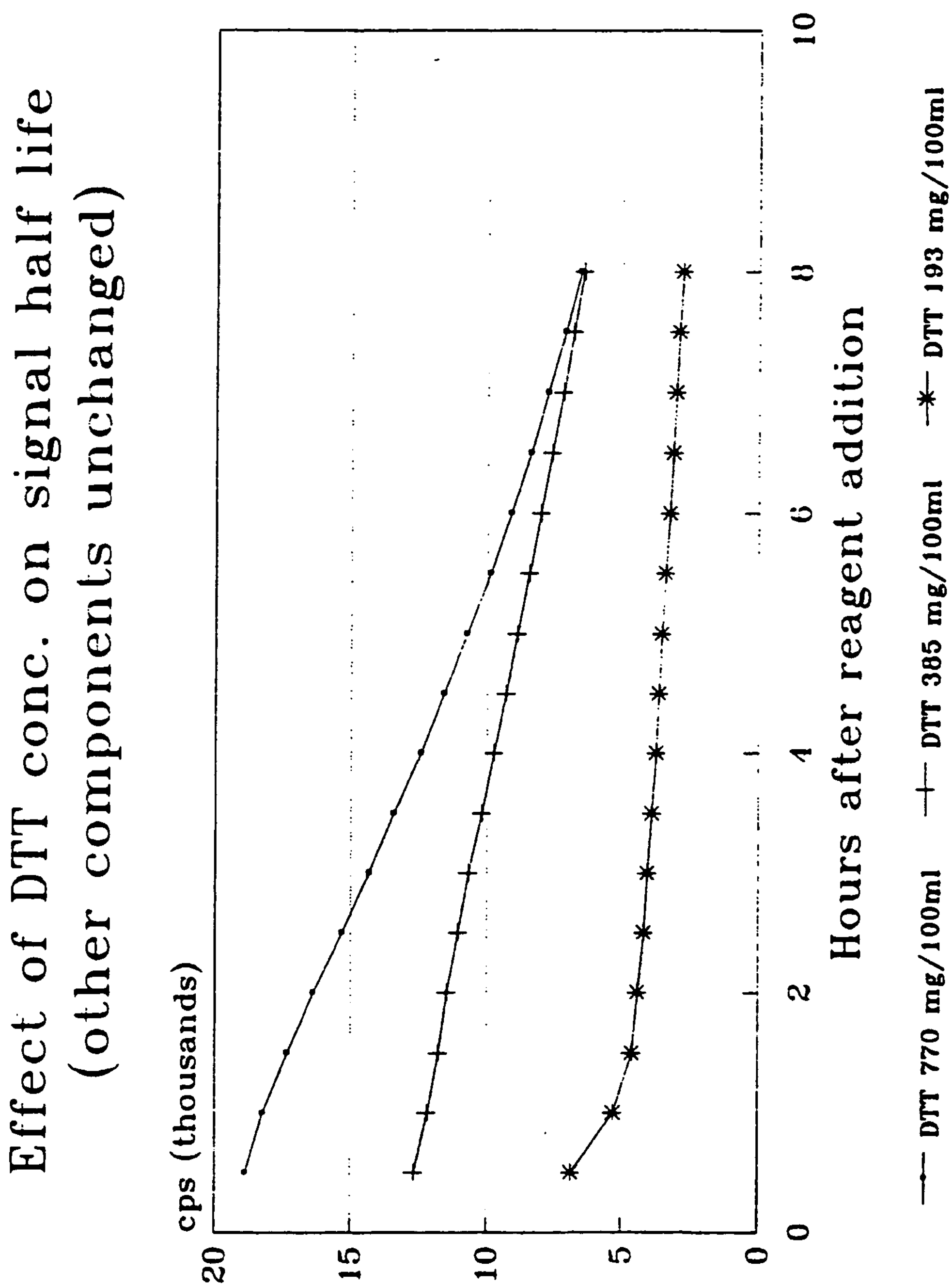

Effect of DTT conc. on signal half life
(other components unchanged)
cps (thousands)
20
15
10
5
0
0
2
4
6
8
10
Hours after reagent addition
DTT 770 mg/100ml
DTT 385 mg/100ml
DTT 193 mg/100ml

**Figure 2**

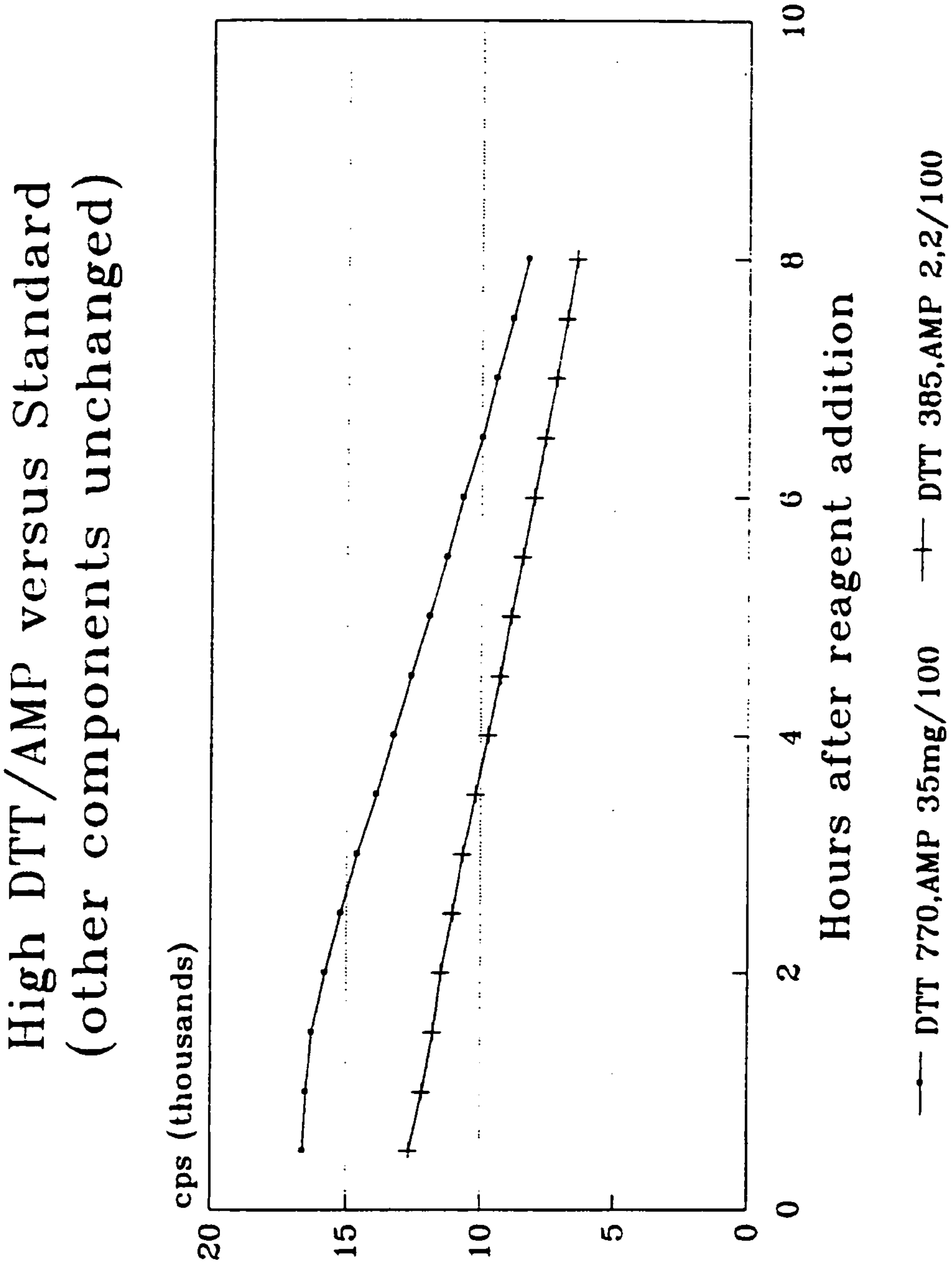

High DTT/AMP versus Standard
(other components unchanged)
cps (thousands)
20
15
10
5
0
0
2
4
6
8
10
Hours after reagent addition
DTT 770,AMP 35mg/100
DTT 385,AMP 2,2/100

Figure 3

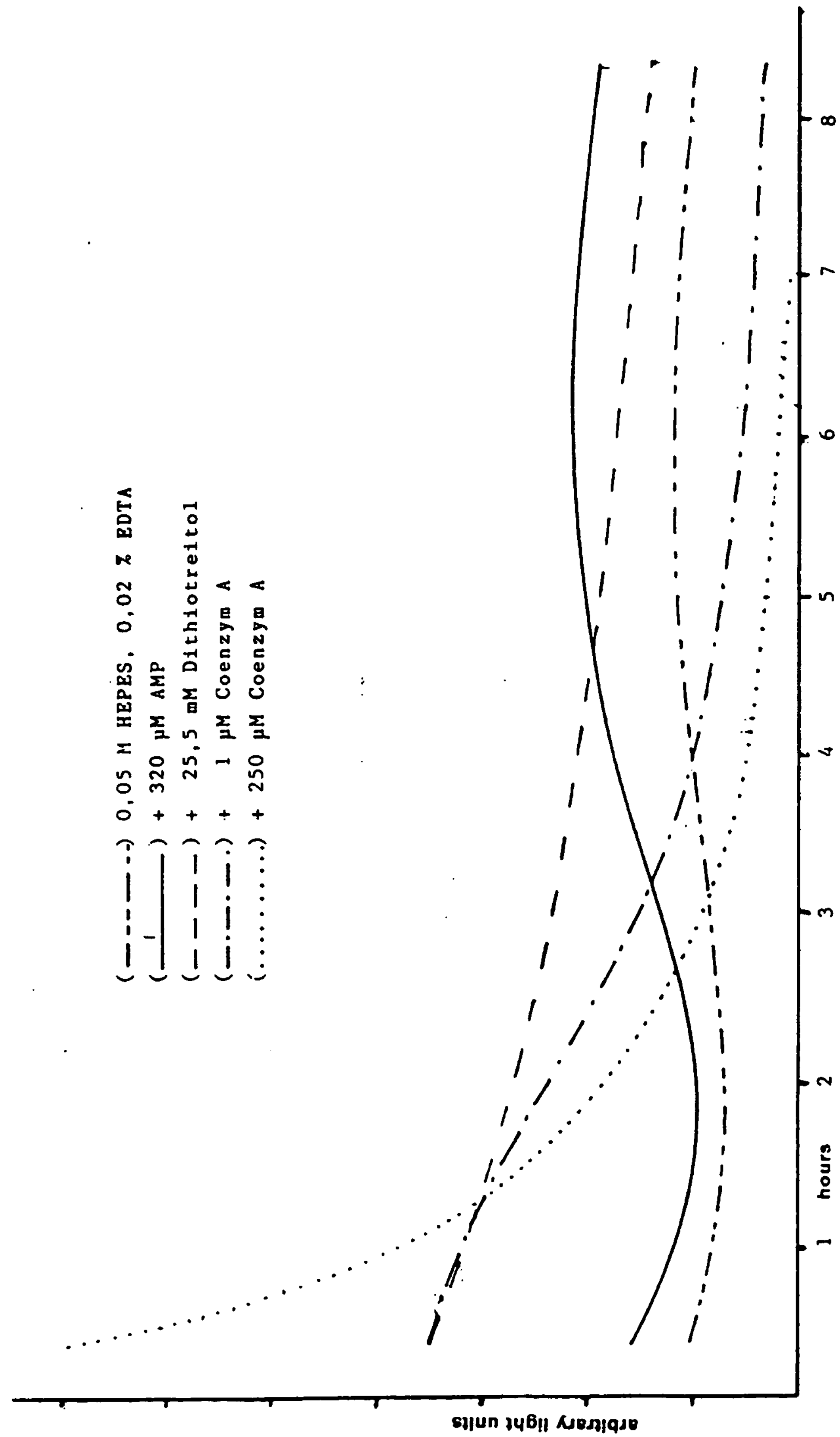

(——-·-·——-·) 0,05 M HEPES, 0,02 % EDTA
(———————) + 320 µM AMP
(— — — —) + 25,5 mM Dithiotreitol
(—·—·—·) + 1 µM Coenzym A
(··········) + 250 µM Coenzym A
hours
1
2
3
4
5
6
7
8
arbitrary light units

Figure 4

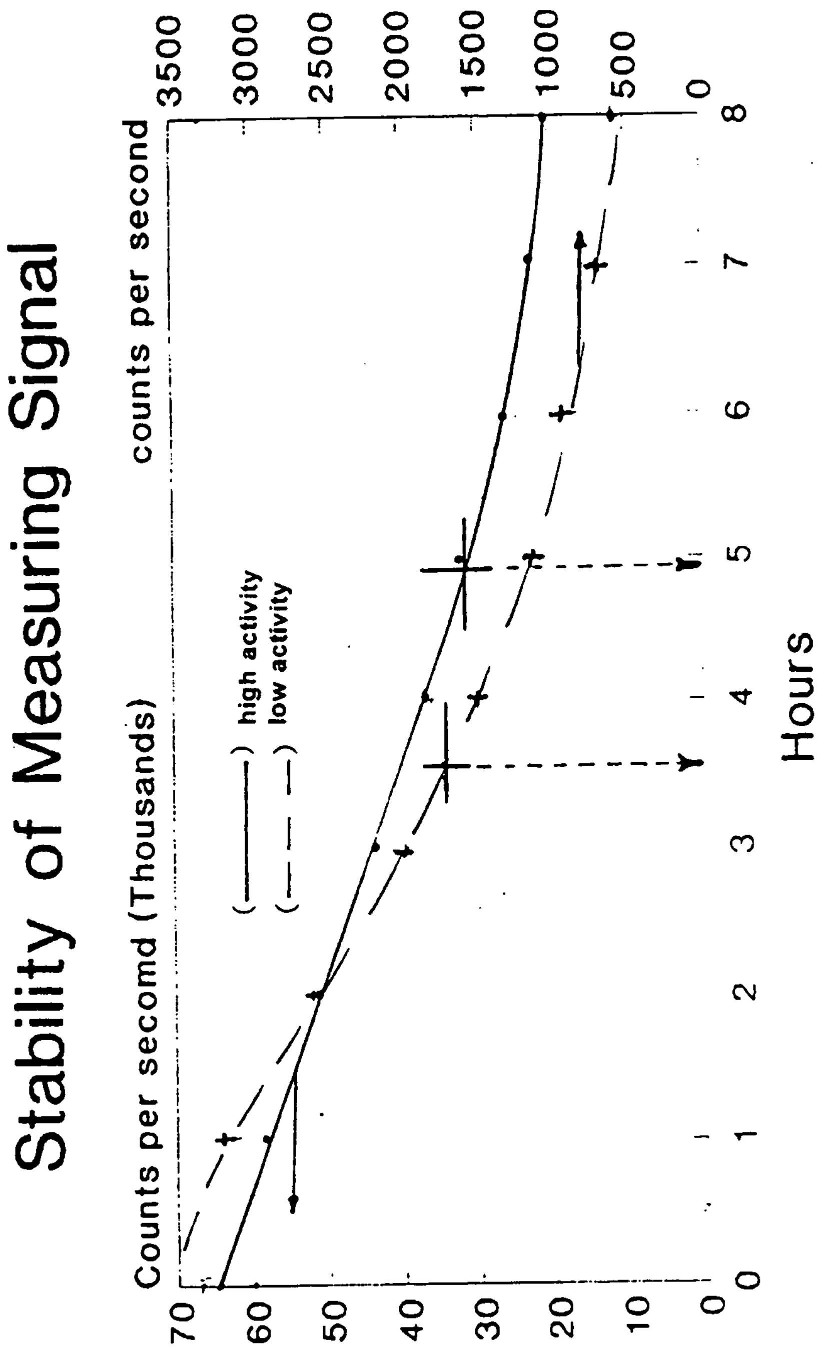

Stability of Measuring Signal
Counts per secomd (Thousands)
counts per second
high activity
low activity
Hours
70
60
50
40
30
20
10
0
3500
3000
2500
2000
1500
1000
500
0
1
2
3
4
5
6
7
8

Figure 5

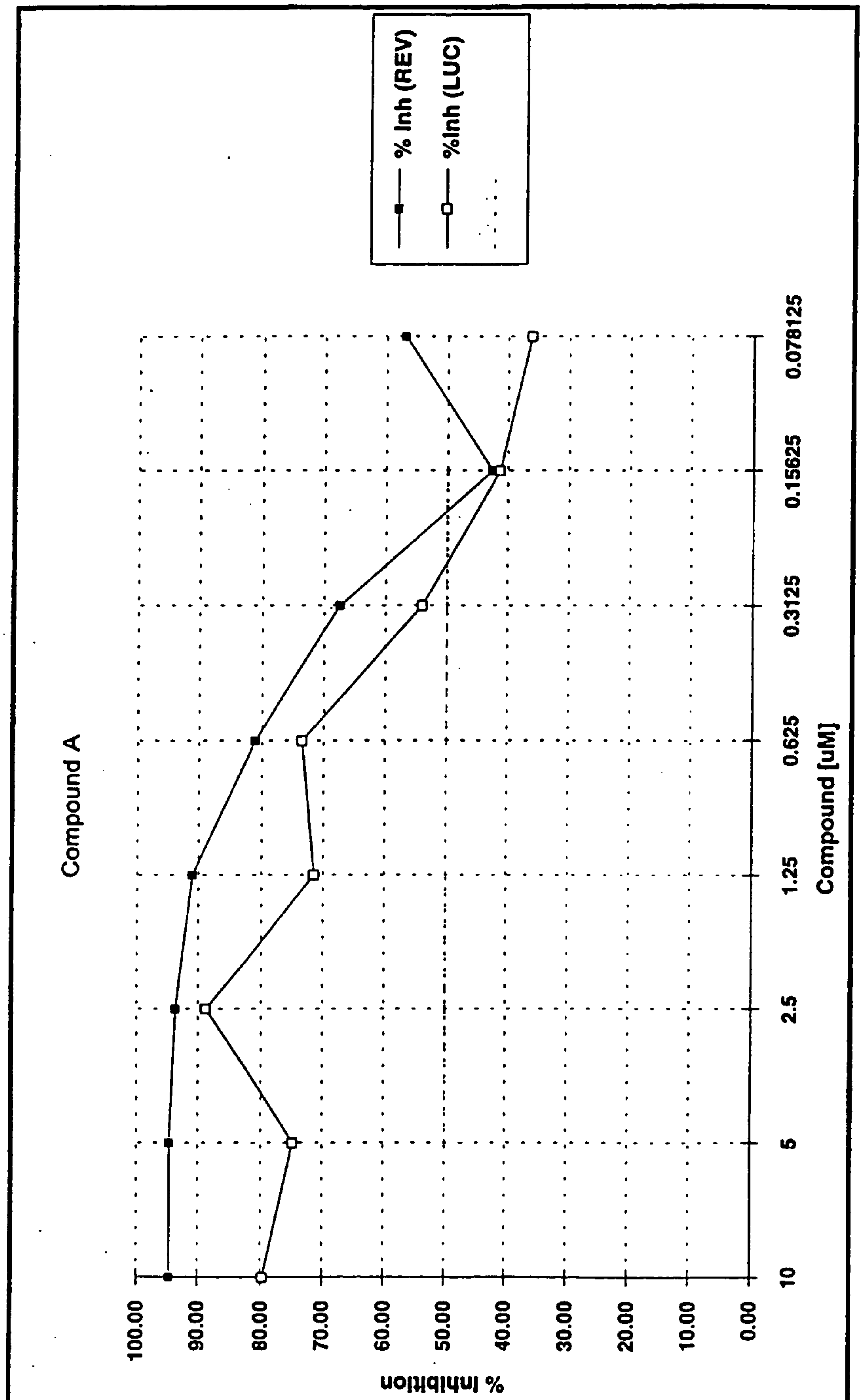
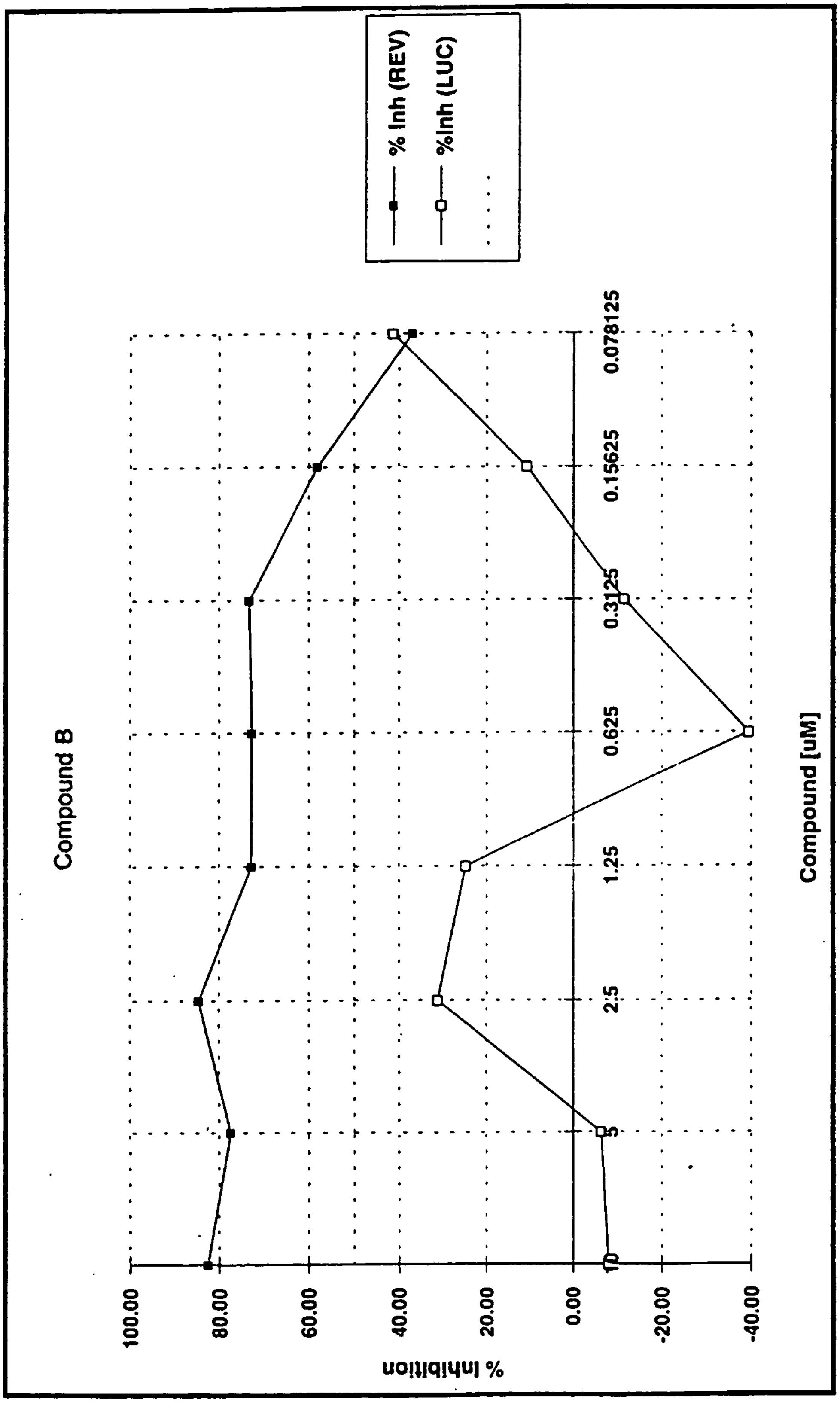

Compound A
% Inhibition
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
10
5
2.5
1.25
0.625
0.3125
0.15625
0.078125
Compound [uM]
% Inh (REV)
%Inh (LUC)

Figure 6



Compound B
% Inh (REV)
%Inh (LUC)
% Inhibition
100.00
80.00
60.00
40.00
20.00
0.00
-20.00
-40.00
10
5
2.5
1.25
0.625
0.3125
0.15625
0.078125
Compound [uM]


Figure 7

European Patent Office

# EUROPEAN SEARCH REPORT

Application Number
EP 94 10 2080

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (Int.Cl.5) |
|---|---|---|---|
| X | EP-A-0 015 437 (BOEHRINGER MANNHEIM GMBH)<br>* page 4, line 26 - page 7, line 2; claims * | 1-3 | C12Q1/66 |
| Y | EP-A-0 022 757 (LKB-PRODUKTER AB)<br>* the whole document * | 1-3 | |
| Y | EP-A-0 299 601 (A/S N. FOSS ELECTRIC)<br>* column 8 - column 10 * | 1-3 | |
| A | EP-A-0 516 443 (ELI LILLY AND COMPANY)<br>* column 9; claims * | 10-12,18 | |
| A | CLINICAL CHEMISTRY<br>vol. 28, no. 8 , August 1982<br>pages 1742 - 1744<br>I.BJÖRKHEM ET AL. 'A Simple, Fully Enzymic Bioluminescent Assay for Triglycerides in Serum'<br>* page 1742 * | 1,3,4 | |
| | | | TECHNICAL FIELDS SEARCHED (Int.Cl.5) |
| | | | C12Q |

The present search report has been drawn up for all claims

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| THE HAGUE | 9 May 1994 | Hitchen, C |

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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L : document cited for other reasons

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